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the Timing of High Dose Therapy for Breast Cancer

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13. ABSTRACT (Maximum 200 words) For drugs that interact with DNA, measures of DNA damage can assess the intracellular availability of active drug at a critical molecular target. Measurements of DNA damage should reflect the integrated effect of all resistance factors, including both recognized mechanisms and uncharacterized mechanisms. Thus, molecular measures of DNA damage could provide an important tool for elucidating the time course of complex changes in resistance factors. Motivated by a recent clinical trial that demonstrated better survival when the interval between induction chemotherapy and high dose therapy was prolonged, this project is using measures of DNA damage in patient blood cells to determine whether induction chemotherapy causes transient changes in resistance. Findings indicate cyclophosphamide, cis-platin, and BCNU each produce DNA damage that can be measured in a dose dependent manner. At low doses each agent causes a similar pattern of breakage, while at high doses their pattern of damage could be distinguished. Both lymphocytes and bone marrow cells from patients can be routinely analyzed, allowing study of changes in sensitivity in multiple tissues. It is feasible to use the procedure to study whether induction therapy has a transient effect on resistance to high dose therapy.				
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FOREWORD

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 27 July 99
PI - Signature Date

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5. **Introduction** The immediate goal of this work is to contribute to the understanding of the unexpected schedule dependency observed between induction and high dose therapy in a recent trial of high dose therapy with cyclophosphamide, BCNU, Cisplatin and marrow or autologous stem cell support (CPB-ACS). That trial unexpectedly found that CPB-ACS was more successful when used at relapse (overall survival 40%) than immediately after induction therapy (overall survival 20%). We proposed to use molecular methods to determine whether the levels of DNA damage caused by the CPB change when it is administered at different times after induction therapy. Understanding the basis for the schedule dependency of this treatment program would directly facilitate development of more effective scheduling for this and potentially other high dose chemotherapy programs. In a broader context, work done here to develop measurements of DNA damage and molecular dose for clinical oncology will provide a rapid way to measure the effects of alternative treatment programs. These measurements would provide an approach to develop optimal treatment protocols with many fewer patients much more rapid timeframe than could be achieved using clinical outcomes.

6. Body of Report

The initial goals of the project (Tasks 1-3 of our Statement of Work) included obtaining specimens and development of assays as presented below. To date our progress is as follows:

Obtain specimens of tumor, blood cells, and other tissues from high dose chemotherapy studies for analysis. During the past year over 40 bone marrow and 200 peripheral blood specimens were accrued to the repository that will be used for this study. Mononuclear cell fractions from these specimens are isolated using ficoll gradients, and, except as noted, the specimens catalogued and stored in the vapor phase of liquid nitrogen. These specimens were used for the assays described below or have been archived for future analyses.

Further develop assays for DNA adducts formed by the chemotherapeutic agents under study in order to optimize them for studying in vivo effects in patient specimen and development of in vitro approaches to studying adducts in short term primary culture. Studies performed to date have emphasized the use of the Single Cell Gel Electrophoresis (SCGE) assay to monitor levels of DNA adducts. This assay is highly sensitive and can be performed on small numbers of cryopreserved cells. For the *in vitro* studies required for this project, this technology allows analysis at multiple dose levels and replicate assays that would not be feasible with other approaches. Studies were performed with a

cell line (MCF7 breast cancer cells, provided by the Tissue Culture Core of the Karmanos Cancer Institute) and peripheral blood lymphocytes and bone marrow cells from patients. Exposures to chemotherapeutic drugs was standardized at a 2 incubation hours at 37°C with various concentrations of 4-OH cyclophosphamide, cisplatin or BCNU.

The assay involves isolation of cells, embedding cells in agarose, lysing cells to remove membrane and proteins leaving DNA, denaturing DNA with high pH, electrophoresing cells, neutralizing, drying, and staining cells with a DNA stain. When performed under alkaline conditions, adducts and other alkali labile lesions are converted to breaks, such that the assay reflects levels of DNA adducts. Nucleoids with intact DNA remain circular after electrophoresis. Cells with DNA breakage reveal a brightly fluorescent circular head and a tail of damaged DNA that has electrophoreses faster than the intact DNA. These units resemble comets, hence the term "comet assay" has been applied widely to the procedure (1-3). The extent of damage is quantified by image analysis.

For most analyses the SCGE assay was performed under alkaline conditions using an adaptation of the method of Singh et al(4). To summarize the method, treated cells were suspended in 0.5% low melting point agarose in PBS at 37°C. 75 ul cell suspension was pipetted onto a frosted glass microscope slide pre-coated with 75 ul 1% normal melting point agarose and covered with a coverglass. After gelling for 5 min at 0°C, the coverglass was gently removed and a third layer of 75 ul LMP agarose was added and covered again with a coverglass. After gelling for 5 min the second time the

slide was put in a tank filled with the lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, NaOH to pH 10.0 , 1% Triton X-100, and 10% DMSO) at 4°C for at least 4 hours. The slides were next placed in a horizontal electrophoresis tank containing 300 mM NaOH and 1 mM EDTA for 20 min at 4°C to allow for unwinding of the DNA prior to the electrophoresis at 25 V (1 V / cm, 300 mA) for 30 min. The slides were then washed 3 times for 5 min with cold neutralization buffer, 0.4 M Tris-HCl, pH 7.5.

Finally the slides were immersed in ethyl alcohol for 5 min before staining with Cyber Green 1. For these procedures plastics were from Corning (NY,USA) RPMI 1640 medium with phenol red and fetal bovine serum were obtained from ATCC, Manassas (VA, USA) The trypsin, salts, and buffers used in the lysis solution and the electrophoresis buffer were from Sigma, St. Louis (MO, USA).

Levels of DNA damage are determined by staining with Syber Green and stained nucleoid were examined using a Zeiss epifluoresence microscope with 40 x Plan-neoflur objective and a 50 w mercury power source and appropriate filter. The microscope was attached to an imaging system consisting of a intensified CCD camera and a IBM compatible PC computer with a KOMET Assay Software (Kinetic Imaging, LTD). From each slide typically 50 images were analyzed sequentially; only overlapping cells were omitted from analysis. The image analysis algorithm used first applied an edge filter to define the limits of the comet, then subtracted the background (defined as the image intensity at the edge of the comet), and subsequently formed head and tail distributions for analysis. The Olive Tail moment, defined as the product of the amount of DNA in the

tail multiplied by the tail length (distance between edge of comet head and end of tail), was used as a measure of the amount of damage in individual cell (5, 6). Further details of the experimental method are presented in draft of our standard operating procedure ma

We have previously shown that CPB treatment *in vivo* causes damage readily detectable by the alkaline SCGE. A limitation is that the damage appears similar for different drugs, so that *in vivo* it is likely to be difficult to discern the effects of individual drugs used for combination therapy. This problem - the relative non-specificity of the endpoint - is not a factor when studying the effects of agents *in vitro*, so we are using this approach to determining levels of DNA damage. Also, we conducted pilot studies of several methods for isolating DNA for chemical assays of damage, obtained adduct standards and HPLC columns suitable for chemical analysis, and procured components of instrumentation needed for chemical assays. The DNA isolation work indicates that we will obtain about 1 ug of suitable DNA per ml of blood. While this will provide sufficient material for chemical analyses *in vivo*, where a single isolation of DNA will be used for several assays, the quantities required and difficulty isolating very small amounts of DNA will preclude conducting dose-response curves with replicate analyses *in vitro* (each *in vitro* treatment requires a separate DNA isolation.) These considerations prompted focus on developing the *in vitro* SCGE assays.

For this project we conducted a series of *in vitro* experiments establishing dose-response relationships for DNA breakage induced by each of the individual agents used for CBP. Each agent induced DNA breakage at doses comparable to estimated

AUCs that would be achievable in vivo with CPB therapy. Qualitatively, at low doses the pattern of breakage was grossly similar for each agent. At high doses the pattern of damage differed for each agent. These patterns were consistent for most of the cells assayed. The patterns were also similar to those previously described in the literature for comet damage. These patterns are presented in Figure 1 (Appendix).

In these studies damage caused by high doses of:

- Cisplatin contracted the cells, consistent with its ability to cause DNA crosslinks and patterns of Comet damage previously seen with crosslinking agents.
- BCNU caused a pattern suggesting diffusion of relatively constant molecular weight fragments prior to electrophoresis and displacement of those fragments during electrophoresis, a pattern shown to indicate apoptosis in a recent series of studies.
- 4-hydroxy cyclophosphamide (4-HC) caused broad diffusion of the DNA, a pattern previously linked with necrosis.

Quantitatively, levels of DNA damage as measured by Olive tail moment (product of DNA fluorescence in the tail and tail length) increased with increasing concentrations of each drug. Each agent caused damage in a dose-dependent manner in both MCF7 cells and primary lymphocyte cultures, with flattening at very high doses of BCNU and 4OH-CP (Figures 1 -7). There was a reversal at high doses of Cisplatin consistent with its action as a crosslinker (Figures 2 and 5). Dose dependent measurements of damage can be made for a broad dose range, avoiding flattening at high doses.

In addition to these studies of MCF7 cell lines and primary lymphocytes, we analyzed specimens of mononuclear cells isolated by ficoll gradients of bone marrow

aspirates from patients prior to CPB therapy available from our repository. These specimens were cryopreserved using a step-down freezer and stored in the vapor phase of liquid nitrogen for less than 3 months. Specimens were rapidly thawed, washed, incubated with BCNU for two hours, and analyzed by the alkali SCGE assay as described in Figure 8 and 9. Damage was readily and quantitatively detected in these cells, indicating they will allow analyses of alterations in resistance after induction therapy.

We also assayed specimens available from a repository in which specimens were obtained and processed in a similar manner but stored at -80 deg C for over 2 years. These specimens had a very high background of damage in the pretreatment samples, levels that precluded their use for studying chemotherapy induced damage (data not shown). A variant of the SCGE assay, however, has allowed us to use the several year old cells to study both *in vivo* effects and *in vitro* changes in sensitivity to damage. This assay uses neutral conditions instead of alkali for lysis and electrophoresis: lysis is done in 30 mM EDTA, 0.5% Sodium Dodecyl Sulfate at pH 8.3, and electrophoresis is conducted in TBE buffer (90 mM Tris base, 90 mM boric acid, 2 mM EDTA). We have determined that this assay is extremely sensitive to crosslinking. As in the standard assay, cross-linkers contract the cells, resulting in smaller tail moments. This occurred in 8 of 10 patients following CPB therapy. It also occurred in all instances following *in vitro* exposure to CisPlatin (Table 1).

7. Key Research Accomplishments

- Assays for measuring transient changes in resistance to CPB therapy following induction therapy were established using procedures that use very small numbers of cells. This technique will allow studies of multiple doses and replicate assays for the *in vitro* studies required for this project.
- Assays demonstrated that these procedures could be used for both lymphocytes and bone marrow cells from patients
- A modification of the assay can be used to study transient changes in cells stored for prolonged periods

8. Reportable Outcomes

Land, S.J., Bacsó, Zs., Klein, J., Eliason, J.F., and Everson R.B. Quantitative methods for examining the drug resistance phenotype of micrometastatic cancer cells in bone marrow: DNA damage in response to *in vitro* drug treatment. (Presentated at the Annual Meeting of the American Association for Cancer Research, 1999)

9. Conclusions

- Each of the agents in CPB produces DNA damage that can be measured with the SCG assays in a dose dependent manner
- At low doses all three agents cause a similar pattern of breakage, while at high doses their pattern of damage could be distinguished (Cisplatin contracted the cells, consistent with its ability to cause DNA crosslinks; BCNU caused a pattern suggesting formation of relatively constant molecular weight fragments; cylophosphamide caused fragments of diverse molecular weights.

- Both lymphocytes and bone marrow cells from patients can be routinely analyzed, allowing study of changes in sensitivity in multiple tissues
- It is feasible to use the procedure to study whether induction therapy has a transient effect on resistance to high dose therapy

10. References

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4. Singh, N. P. e. a. A simple technique for quantitation of low levels of DNA damage in individual cells, *Exp. Cell Res.* 1988: 184-191, 1988.
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6. Olive, P. L. and Banath, J. P. Multicell spheroid response to drugs predicted with the comet assay, *Cancer Res.* 57: 5528-33, 1997.

Figures 1-7 Patterns of Damage and Dose Response Curves for SCGE Assays of Agents in CPB

Table 1 SCGE Neutral Assays with Cis-Platin

SOP for Comet Assay

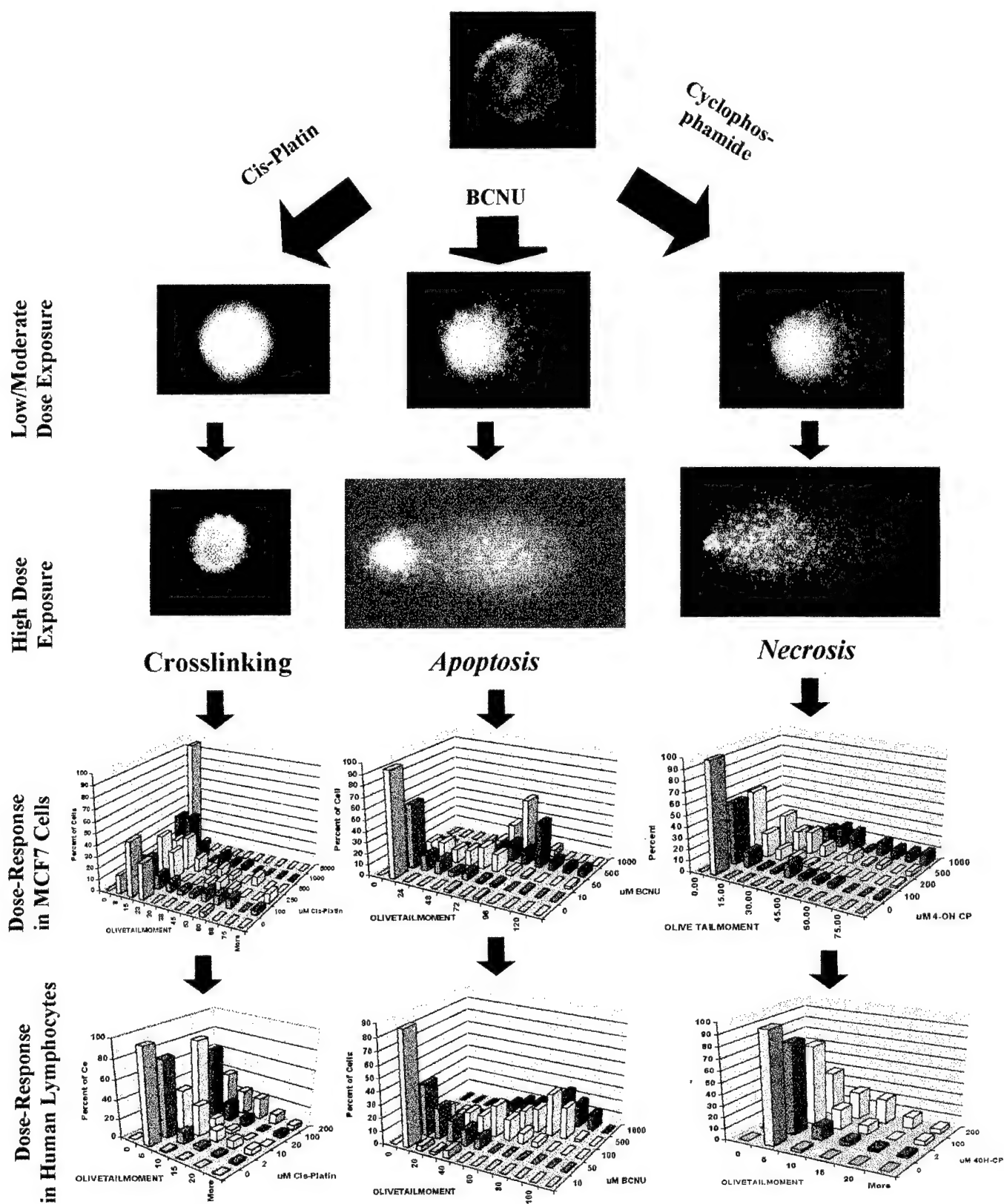


Figure 1. Patterns of Damage and Dose Response Curves for SCGE Assays of Agents in CBP.

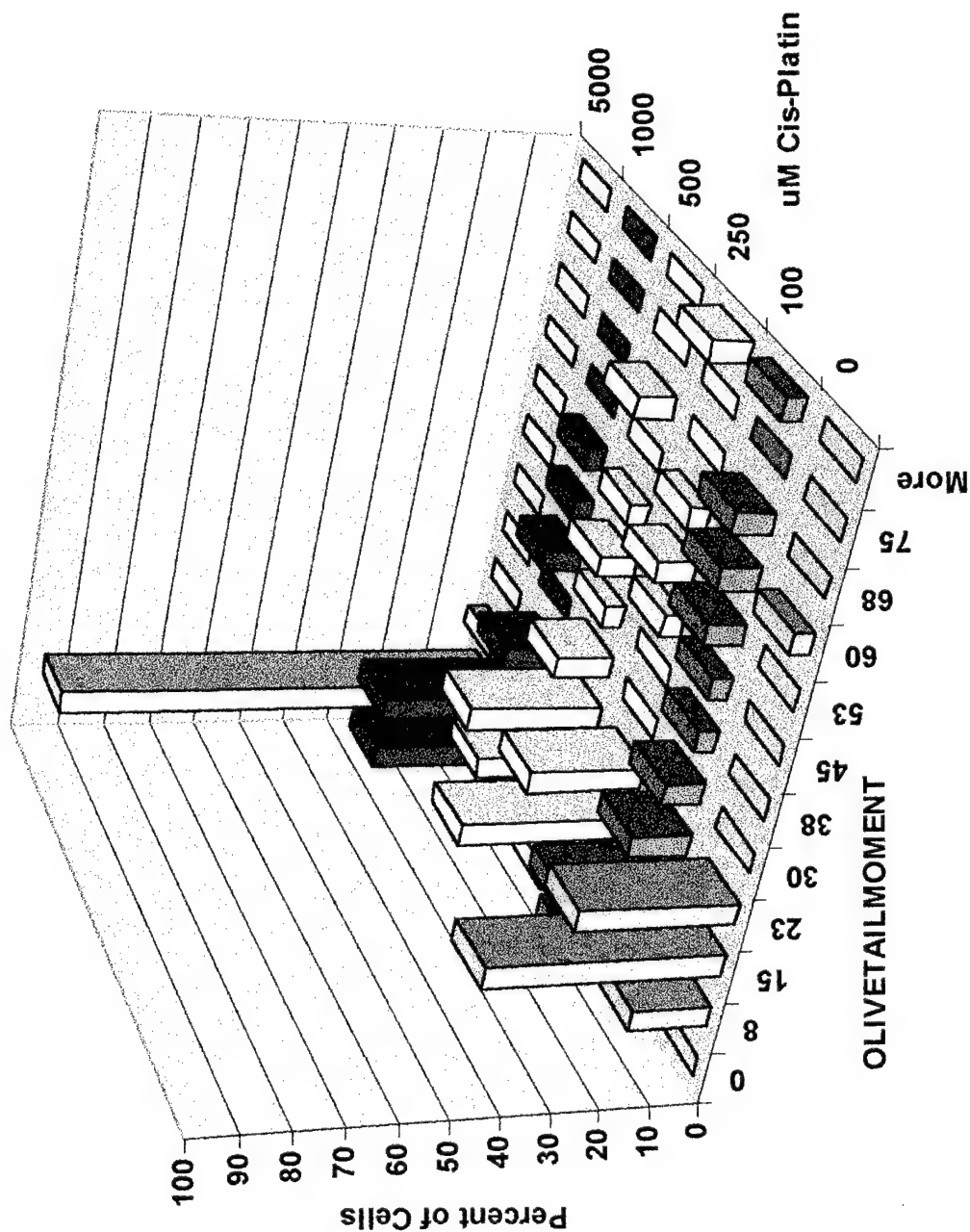


Figure 2. Dose Response Curve for SCGE Assays of MCF7 Cells with CisPlatin. Cells were incubated for two hours at 37 deg C and analyzed by the alkali SCGE assay.

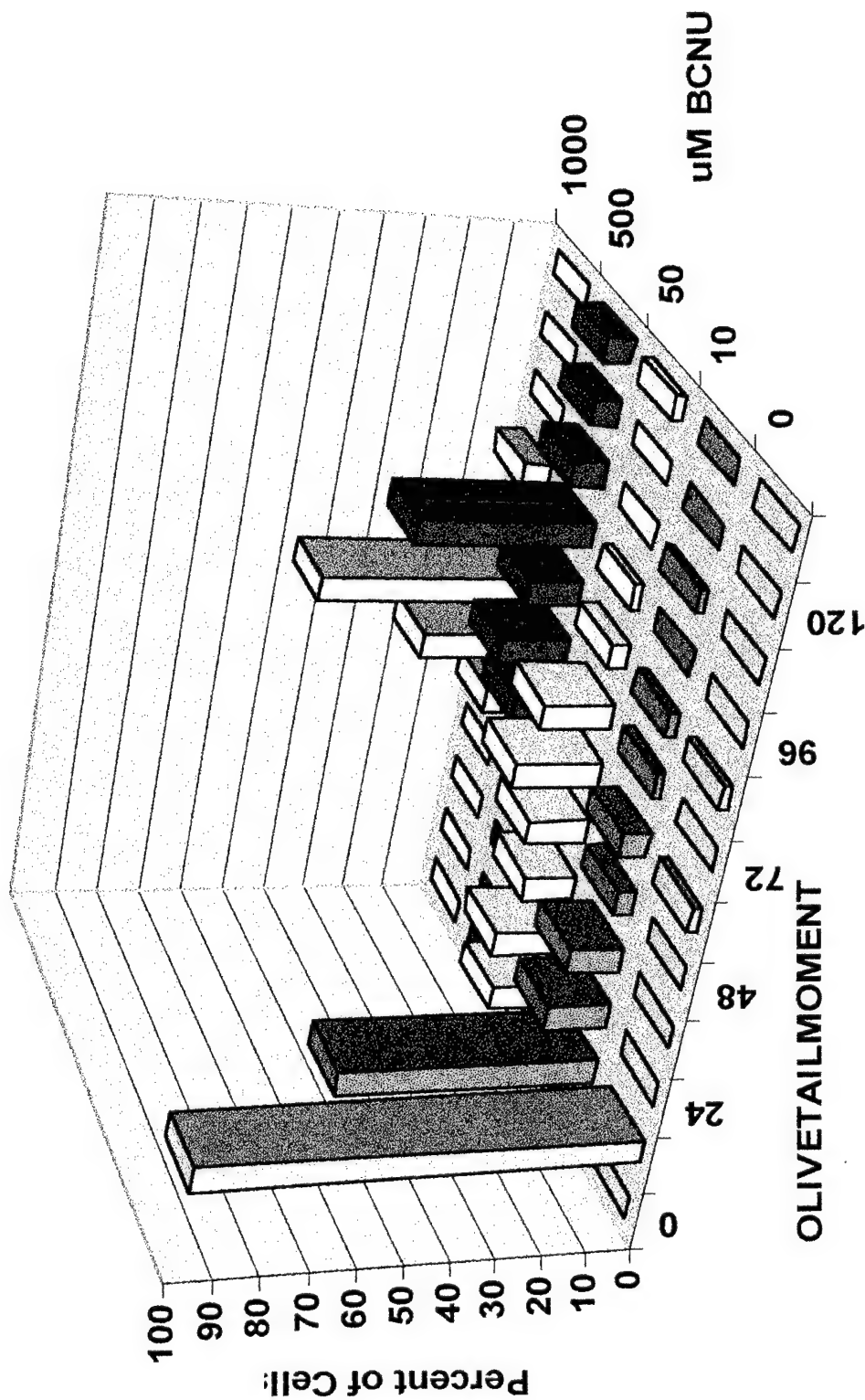


Figure 3. Dose Response Curve for SCGE Assays of MCF7 Cells with BCNU. Cells were incubated for two hours at 37 deg. C and analyzed by the alkali SCGE assay.

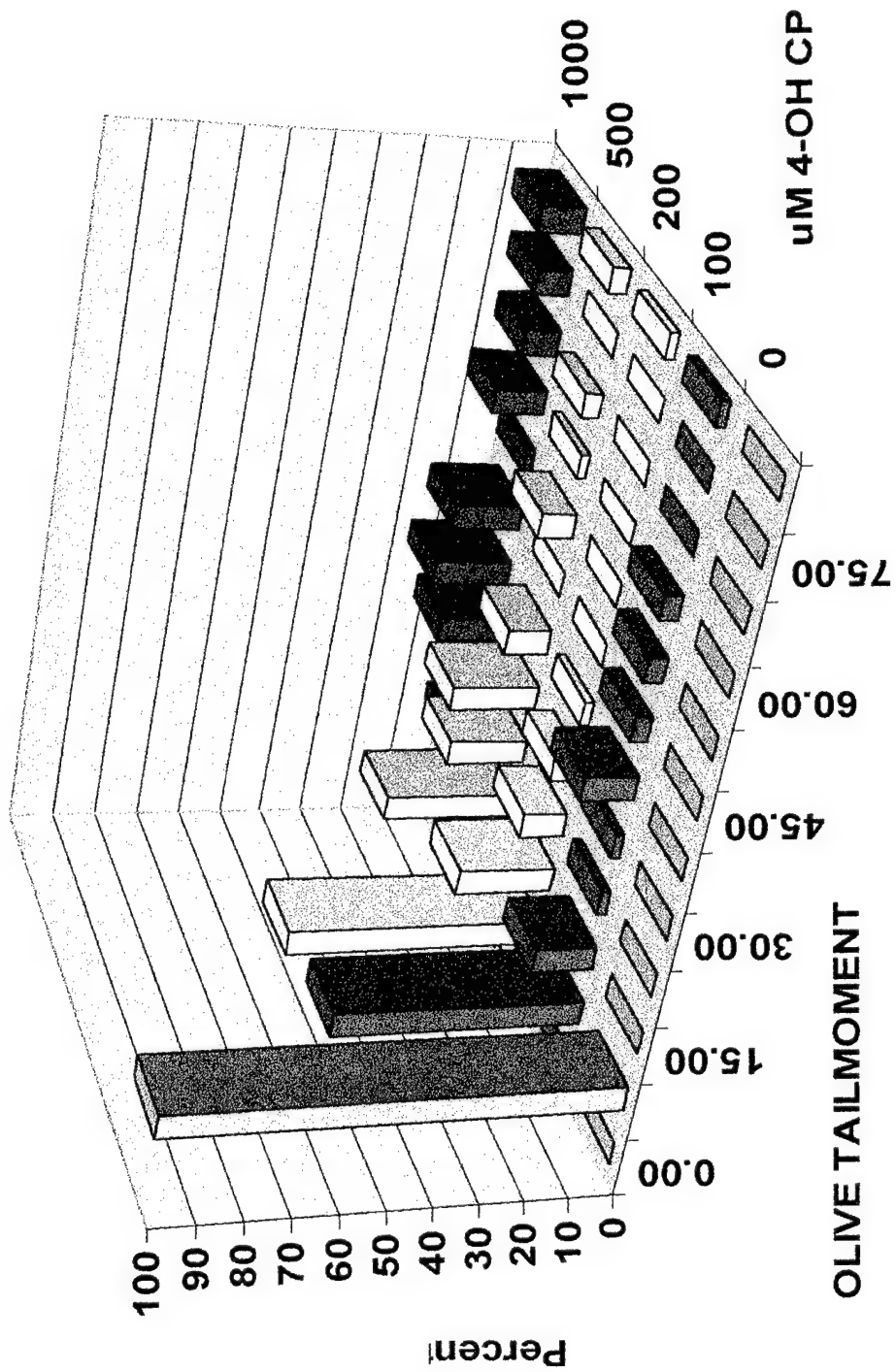


Figure 4. Dose Response Curve for SCGE Assays of MCF7 Cells with 4-OH Cyclophosphamide. Cells were incubated for two hours at 37 deg. C and analyzed by the alkali SCGE assay.

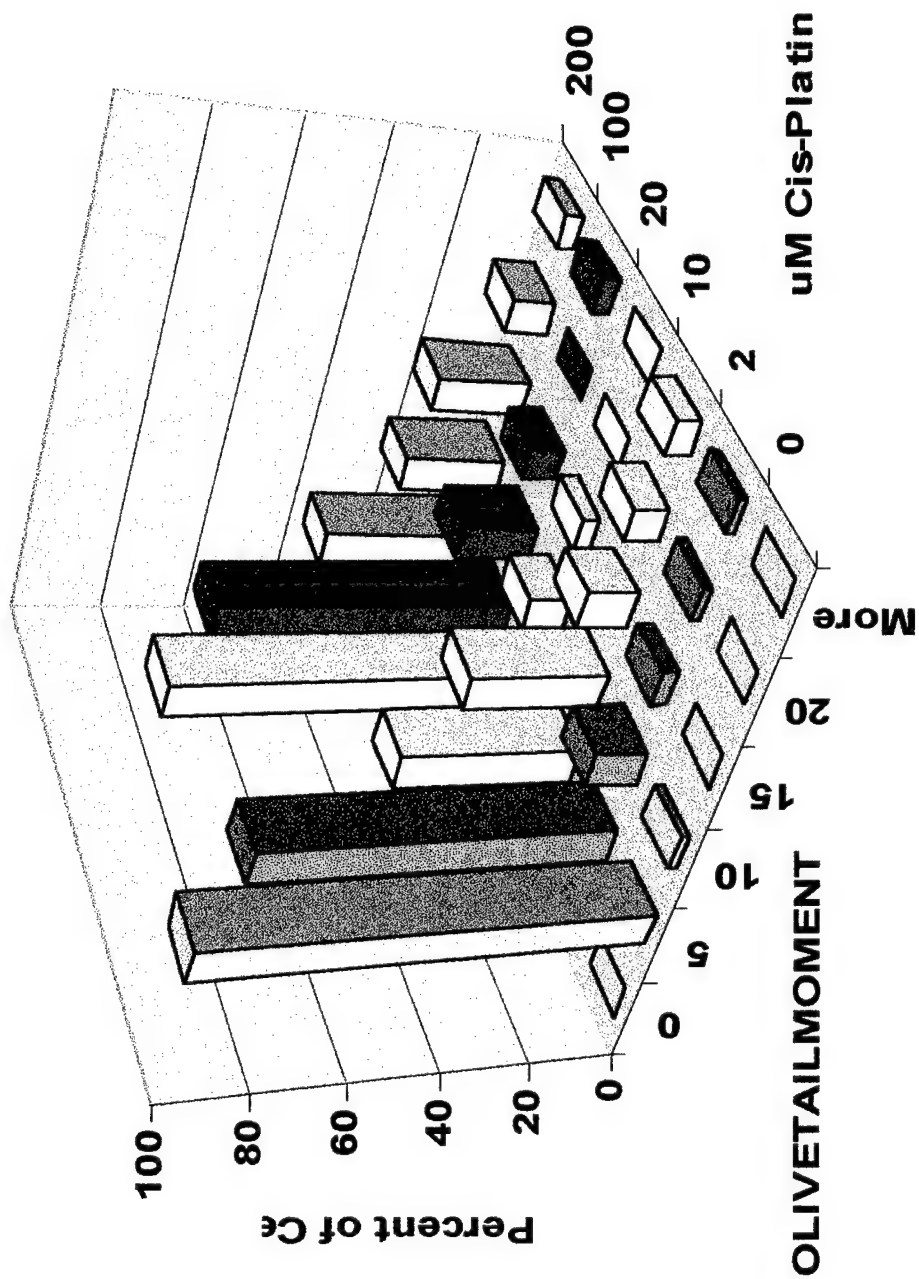


Figure 5. Dose Response Curve for SCGE Assays of human lymphocytes with Cis-Platin. Cells were isolated by ficoll, incubated for two hours at 37 deg. C, and analyzed by the alkali SCGE assay.

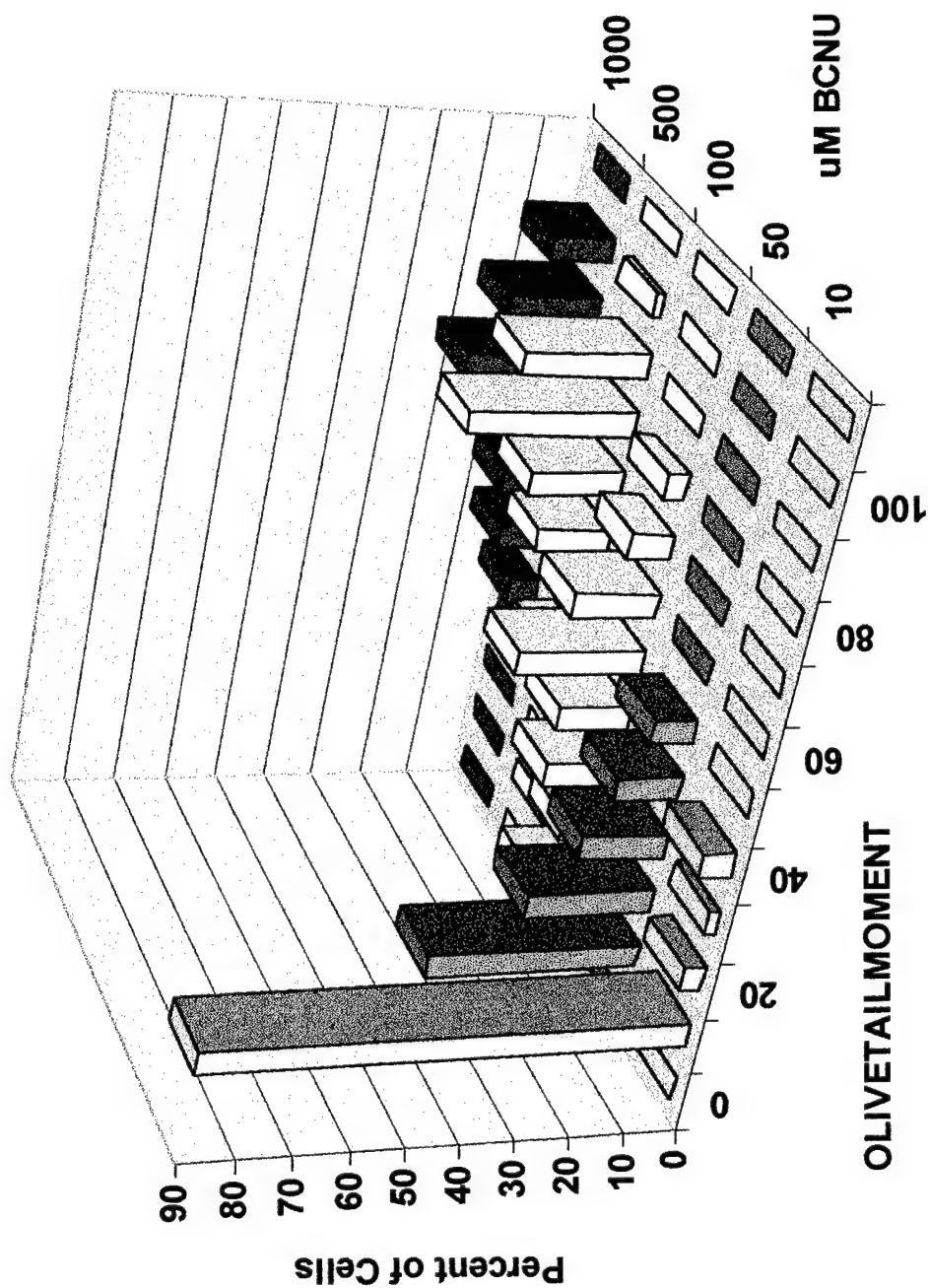


Figure 6. Dose Response Curve for SCGE Assays of human lymphocytes with BCNU. Cells were isolated by ficoll, incubated for two hours at 37 deg. C, and analyzed by the alkali SCGE assay.

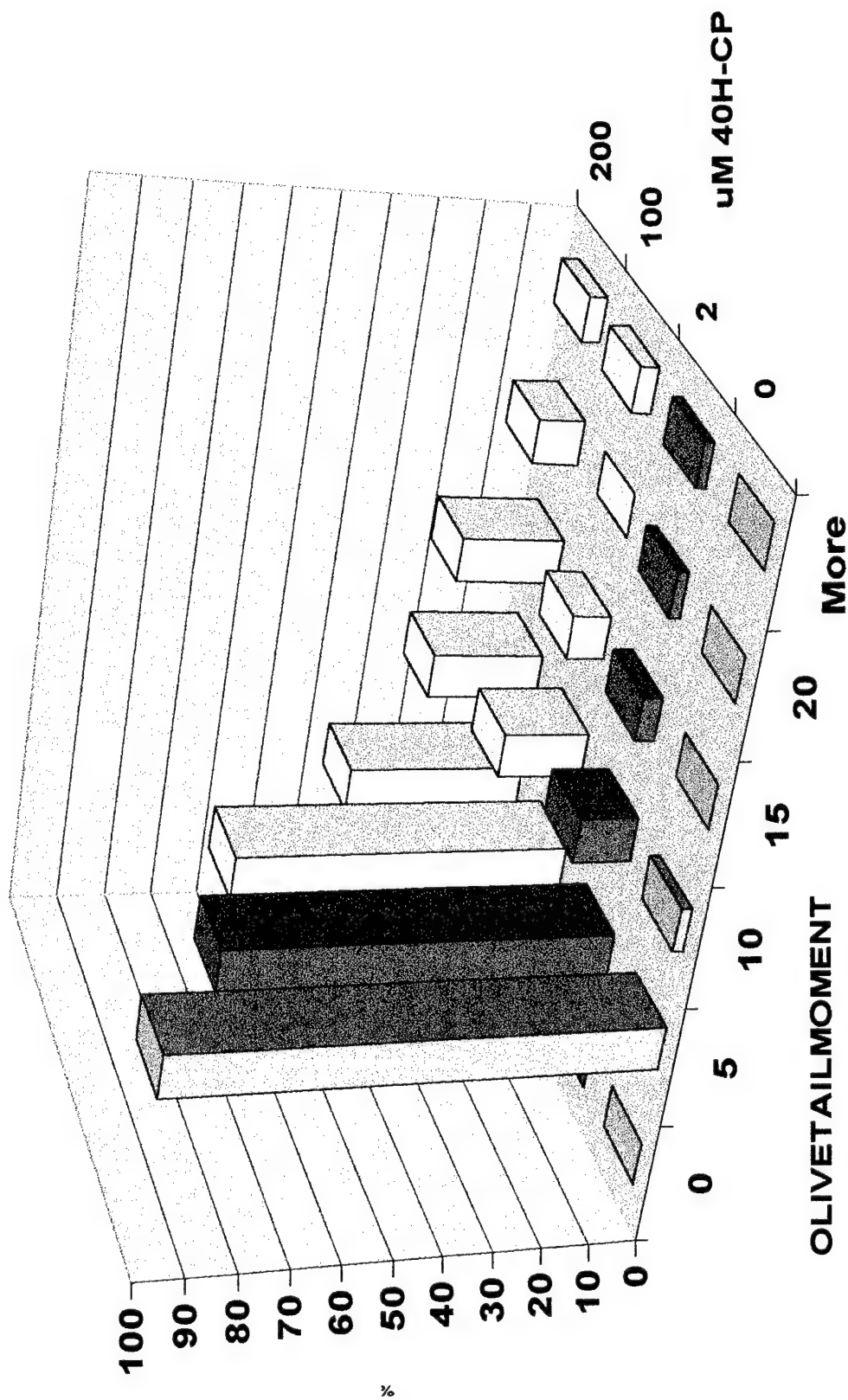


Figure 7. Dose Response Curve for SCGE Assays of human lymphocytes with 4-OH Cyclophosphamide. Cells were isolated by ficoll, incubated for two hours at 37 deg. C, and analyzed by the alkali SCGE assay.

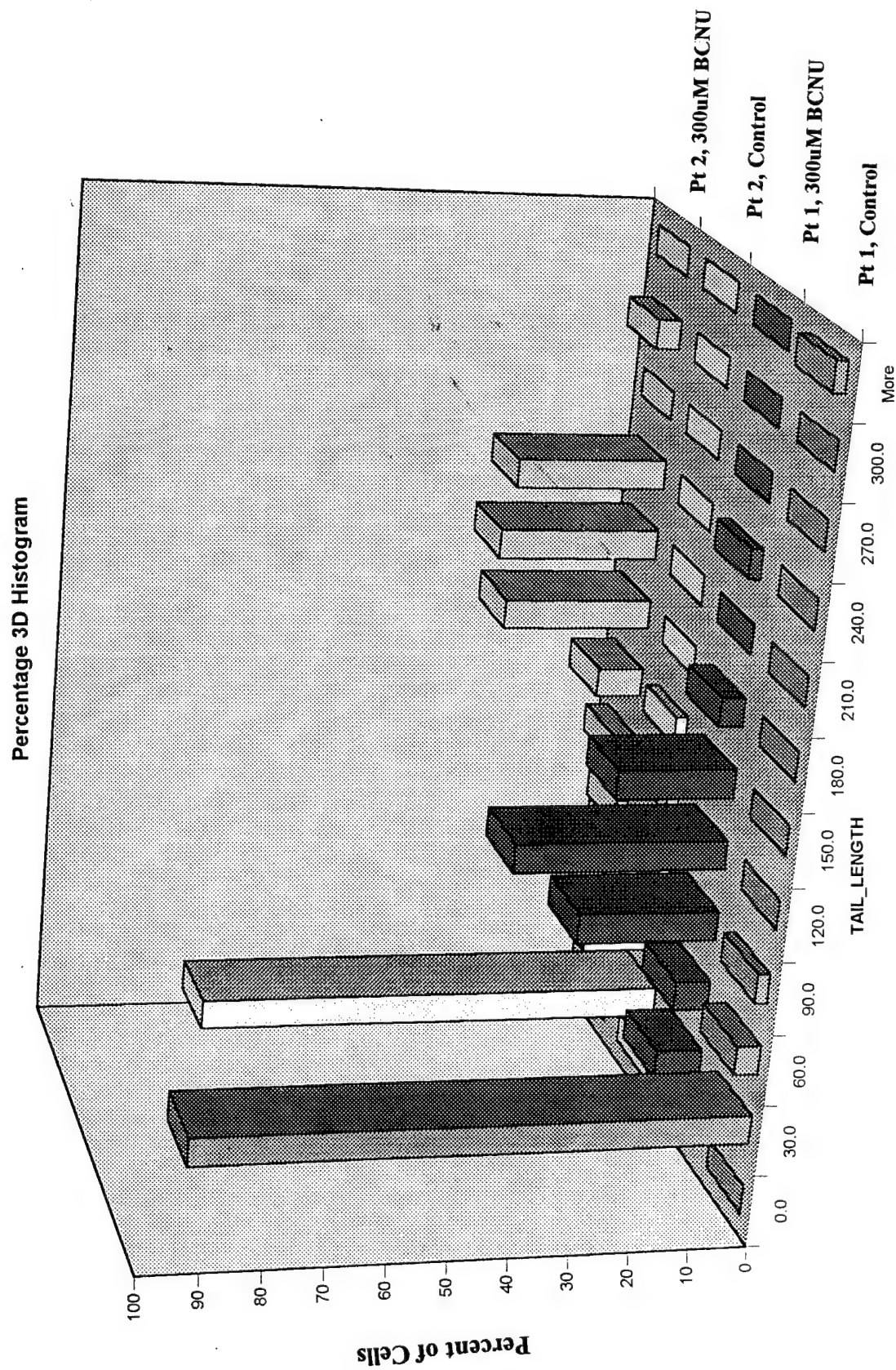


Figure 8. Effect of 300 uM BCNU on bone marrow cells isolated from patients prior to CPB therapy.

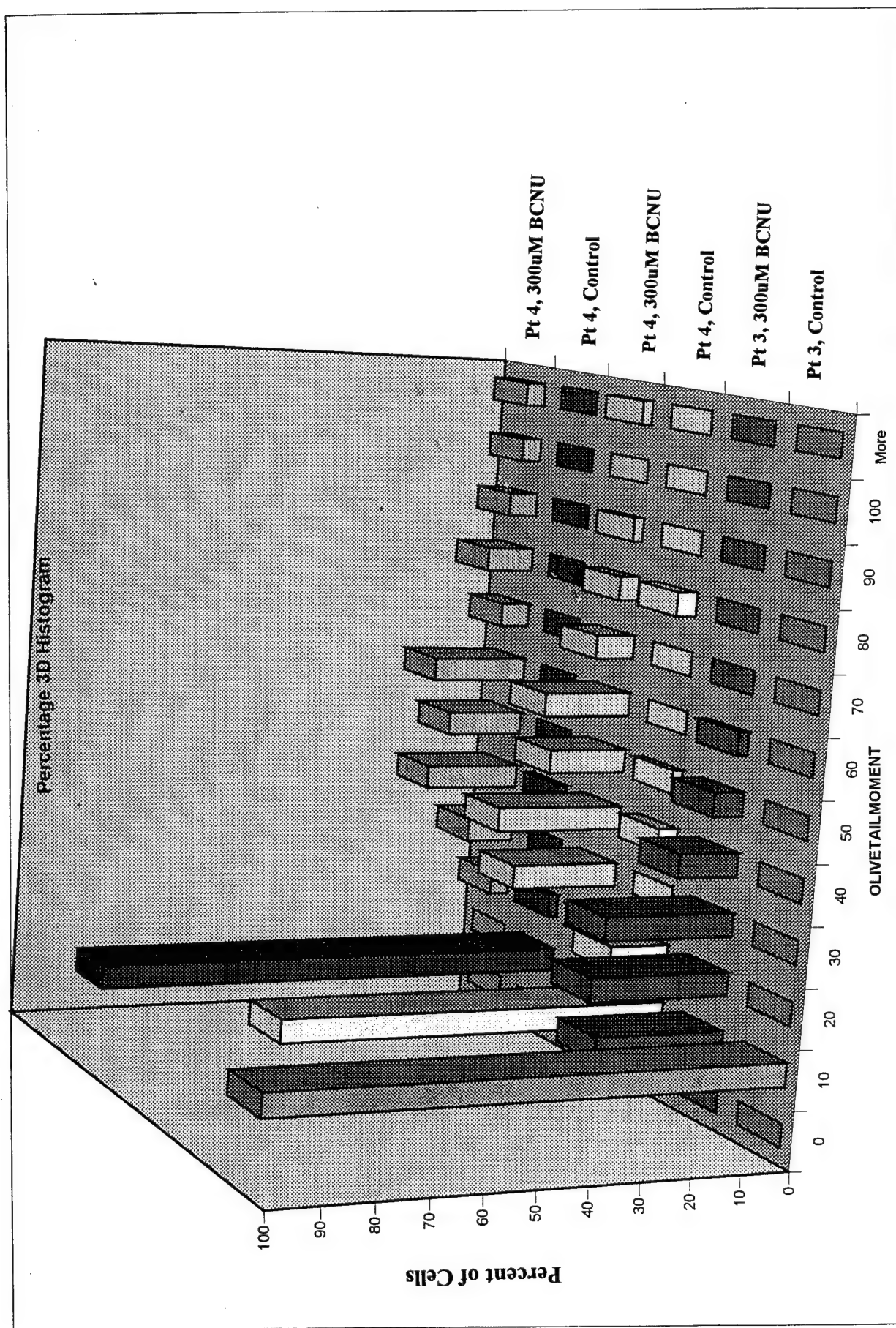


Figure 9. Effect of 300 uM BCNU on bone marrow cells isolated from patients prior to CPB therapy.

Table 1. SCGE Neutral Assays with Cis-Platin

Dose Response Curve for neutral SCGE Assays of lymphocytes obtained prior to CPB therapy or on CPB treated. Specimens were treated with 1 percent DMSO control or 3 to 30 uM Cisplatin. Cells th undergone prolonged storage at -80 deg C were incubated for two hours at 37 deg C and analyzed by neutral SCGE assay. This assay is highly sensitive to crosslinking, which causes a reduction in the ta

Cisplatin (uM)	Mean Tail Moment					
	Pt 1	Pre CPB	Post CBP	Pt 6	Pre CPB	Post CBP
0		57.47	33.41		51.09	38.58
3		57.91	35.06		53.94	36.53
10		23.81	27.05		39.98	23.46
30		3.41	21.01		10.39	5.21
	Pt 2			Pt 7		
0		55.64	39.6		46.89	25.57
3		49.17	50.78		47.47	16.58
10		36.62	12.18		54.14	10.84
30		13.23	7.16		8.98	8.17
	Pt 3			Pt 8		
0		72.59	92.89		58.1	28.27
3		38.78	57.76		45.73	50.63
10		30.47	32.49		10.11	12.64
30		18.33	28.15		4.92	5.77
	Pt. 4			Pt 9		
0		50.12	48.73		45.59	30.26
3		42.36	42.26		35.25	19.29
10		24.93	17.3		19.36	11.15
30		17.2	4.84		15.48	7.24
	Pt. 5			Pt 10		
0		53.96	75.58		61.07	50.02
3		58.28	27.98		61.25	46.59
10		12.82	38.02		51.29	43.19
30		32.15	7.62		20.31	8.42

SOP FOR THE COMET ASSAY

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I. PREPARATION OF REAGENTS

a) Hanks Balance Salt Solution with 20 mM EDTA

- To 400 ml HBSS (Ca^{+2} , Mg^{+2} free), add 3.72 g EDTA set pH to 7.5, q.s. to 500 ml.
- Store at 4 degrees Celsius.
- Use: tissue isolation

b) Phosphate Buffered Saline (Ca^{+2} , MG^{+2} free)

- Dolbecco's PBS - 1L packet, add 3.72 g EDTA set pH to 7.4, q.s. to 1000 ml, filter sterilize.
- Store at 4 degrees Celsius.
- Use:

c) Lysing Solution

- Ingredients per 1000 ml:

MAKE THE DAY BEFORE:

2.5 M NaCl	-	146.1 gm
100 mM EDTA	-	37.2 gm
10 mM Tris	-	1.2 gm
solid NaOH	-	6.5-7 gm (to set pH to 10)
1% Na Sarcosinate	-	10.0 gm

q.s to 890 ml with dH_2O , store at room temperature

DAY OF EXPERIMENT:

1% Triton X-100	10.0 ml
10% DMSO	100.0 ml

NB-remember to refrigerate prior to slide addition for 30-60 min!

d) Electrophoresis Buffer

- 300 mM NaOH / 1 mM EDTA
- Prepare from stock solution:
 - 10 N NaOH - 40 g NaOH to 100 ml CEPB treated water (good for ~2wk).
- For buffer; add 30 ml 10 N NaOH and 2 ml of 0.5 M EDTA to a final volume of 1000 ml in CEPB treated water (make fresh just before use).

e) Neutralization Buffer

- 0.4 M Tris, pH 7.5
- 63.04 g Tris (acid), q.s to 1000 ml with dH_2O
- Set pH using NaOH
- Store in refrigerator

f) Staining Solution

- Ethidium Bromide (10x Stock - 20 ug/ml)
- 10 mg in 50 ml dH₂O
- Store at room temperature
- For 1 X stock - mix 1 ml with 9 ml dH₂O

II. CELL ISOLATION

a) Whole Blood

- Mix 5 ul whole blood with 75 ul Low Melting Point Agarose, layer onto slide.

b) Isolated Lymphocytes

- Mix 20 ul whole blood with 1 ml RPMI 1640 in a micro centrifuge tube, add 100 ul Ficoll below the blood/ media mixture. Spin for 3 min at 2000 xg. Remove 100ul of middle top layer, add to 1 ml media and mix, spin for 3 min to pellet lymphocytes. Pour off supernatant, resuspend pellet in 75 ul Low Melting Point Agarose, layer onto slide.

c) Frozen Lymphocytes

- Thaw frozen Lymphocytes
- Put cells into a 15 ml tube. Add media (type varies) without serum to 15 ml.
- Centrifuge at 100xg for 10 min at 4 degrees Celsius
Using Beckman GH-3.8 rotor with buckets: 650 RPM = 96.5 xg
- Repeat wash with serum free media
- Resuspend in 2 ml Phosphate Buffer Saline
- Hold on ice, prepare for treatment if any.
- Count cells and determine cellular viability.

d) Tissue Isolation (mice)

(liver, spleen, lung, bone marrow, brain, testis, etc.)

- Remove a small piece of the organ, place in 1 ml of cold HBSS with 20 mM EDTA on ice. Mince into fine pieces, let settle, remove 5 ul cell suspension, add to 75 ul Low Melting Point Agarose, layer onto slide, For easily desegregated tissues (e.g. spleen and testes) add two to three ml more HBSS and remove 5 ul for slides. For liver only, mince into large pieces, let settle, aspirate HBSS, add 1 ml fresh HBSS, mince into finer pieces. Add 5-10 ul cell suspension to 75 ul LMPA, layer onto slide. For bone marrow, perfuse femur with one ml cold HBSS into a microtube, remove 5 ul cell suspension for slides.

e) Culture Isolation

- 1. Monolayer cultures - scrape off cells into the culture media in the cell dish using a teflon scraper (Do not trypsinize cells) to yield approximately 1×10^6 cells/ml. Add 5 ul cell suspension to 75 ul LMP Agarose, layer onto slide.
- 2. Suspension Culture - Add 10 000 cells in 10 ul or less volume to 75 ul LMP Agarose, layer onto slide.

III. CELL COUNT USING A HEMOCYTOMETER

After isolation of cells it is important to estimate the concentration of cells you have. The amount of cells needed for each slide is ca 10 000. i.e. in order to know whether you have enough cells for the assay or not, take the number of slides to be made and multiply with 10 000. This value of total number of cells will also give you an estimate of how to dilute your assay sample with cells.

Ex:

Lymphocytes are treated with 3 different dilutions of the H_2O_2 . From each dilution we will layer 4 slides. After counting the # of lymphocytes, we estimated the volume needed from the concentrated solution of cells in order to have 40000 cell/ml in each tube.

- (1) Count cells and determine cellular viability by mixing in a separate Eppendorf tube:

100 ul Trypan Blue solution
60 ul HBSS
40 ul cell suspension

- (2) Count cells of 5 squares of both grid sections.
(3) Count the # of Live (unstained) and Dead (dead) cells.
(4) Then calculate your concentration of cells by taking the total # of cells for all the ten squares and divide by 10. Multiply by the dilution factor of 5. This value times 10^4 is the # of cells per ml of the cell suspension;

Cells/ml = average count of viable cells per square x 5 (dilution factor) x 10^4

Cells/ml = _____ x 5 x 10^4 = _____

Cell viability = total viable cells/total cells x 100

- (6) After cell counts are determined, decide the volume of the cell suspension is needed to put the necessary number of cells into the assay system.

IV. PREPARATION OF TREATMENT CONDITION

Each test tube must have a final total volume of 1 ml. After adding the volume of cells and the 10 ul of treatment solution, aliquot Phosphate Buffer Saline or media to 1 ml. Work in dark to protect cells and drug from light. Avoid creating bubbles.

a) Determining Concentration of Treatment

(1) The concentration of the drug or the solvent treatment to be used should not be more than 1 % of your assay system. That is 10 ul in a 1 ml total volume. Since 1 % is one part in one hundred, your stock treatment solution must be 100 times more concentrated than your desired concentration.

- Ex. 1:

If 300 uM BCNU is the desired concentration for the assay, multiply by 100 to get the value 30 000 uM which is 0.03 M. Next calculate the weight (gr) of drug in solvent needed in order to obtain the 100x conc., using the formulas: $C_1 V_1 = C_2 V_2$, $C = \text{mole}/V$, Mole = molecular weight/gram.

- Ex. 2:

Using hydrogen peroxide (H_2O_2), determine the stock concentration being used. H_2O_2 mw: 34.01 g/mole.

Stock-Sigma: 30.6% = 30,6g/100 ml

$3.6 \text{ g}/0.1\text{L} \times 1 \text{ mole}/34.01 \text{ g} = 8.997\text{M}$

Then calculate the amount needed to obtain your 100x desirable concentration. Typically the volume of H_2O_2 is very small. For more accurate concentration using the final solution volume being 10 ml, take out the calculated volume H_2O_2 in solvent from the prepared tube holding 10 ml solvent and next add the same amount H_2O_2 .

(2) When the solvent and the drug is mixed, action must be quick. Have ready the assay tubes with the cells suspended in growth media ready for reception of drug. After drug is added to each tube, the tubes should be quickly inverted in order not to create a gradient where the cells at the surface are exposed to drug for longer time than cells throughout the assay tube.

(3) Incubate the tubes with treatment at desired conditions of time and temperature.

(4) After incubation, centrifuge at 100 xG for 10 min at 4 degrees Celsius. Using Beckman GH-3.8 rotor with buckets: 650 RPM = 96.5 xG.

(5) Decant the supernatant and resuspend in 0.5% Low Melting Point Agarose. A

volume of 75 ul of LMPA is needed for each slide to be made out of the assay tube.

(6) Layer slides (see section "Preparation of Slides for the SCG assay")

b) Endonuclease

-Treat slides with endonuclease III for 1hr at 37 degrees Celsius in the incubator. Place slides in glass trays that have 1 ml pipettes along the bottom to hold the slides above the surface. Beneath the pipettes a clean kimwipes to hold DEPC water to help humidify the tray. After the slides are carefully placed on the supportive pipettes, use a Pasteur pipette to drop the endonuclease solution on the slides, approx. 1ml per slide. Cover the tray with plastic wrap to keep from drying out.

c) Repair

- For some assays it is interesting to look at the capability of cells to repair after treatment as opposed to cells analyzed right away.. Hence, we allow one set of treated cells layered on slides to sit for 30-60 min at 36 degrees Celsius in a water incubator to allow for any repair

V. PREPARATION OF SLIDES FOR THE SINGLE CELL GEL ASSAY

Prepare 0.5 % Low Melting Point Agarose and Regular Agarose in Calcium and Magnesium free PBS (125 mg in 25 ml). Microwave or heat until near boiling and the agarose dissolves. Aliquot 5 ml samples into scintillation vials and store at 4 degrees Celsius until needed. When needed, briefly melt agarose in microwave or by another appropriate method. Place LMPA vial in a 37 degrees Celsius water bath to cool.

Already prepared slides with one layer of normal melting point agarose in PBS are used for the layering of the cell suspension .

- (1) Make 1% normal melting point agarose in PBS.
- (2) While agarose is hot, dip conventional slide in a coplinjar up to one half the frosted area and gently remove.
- (3) Wipe backside of slide to remove agarose and lay the slide in a tray on a flat surface to dry. The slides may be air dried or warmed for quicker drying.
- (5) Store the slides at room temperature until needed. Avoid high humidity conditions, if possible.

After the assay tubes with cells have undergone incubation with treatment.

(1) Centrifuge at 100 xG for 10 min at 4 degrees Celsius.

Using Beckman GH-3.8 rotor with buckets: 650 RPM = 96.5 xG.

(2) Decant the supernatant and resuspend in 0.5% LMPA. A volume of 75uL (containing 10 000 cells)ul is needed for each slide.

(3) Keep in water bath at 37 degrees Celsius.

(4) Layer slides

Layering of the slides:

(1) Work in dim light to prevent DNA damage.

(2) On to a slide previously coated with agarose and dried, place a cover slip at a 45 degree angle holding it while adding carefully 75 ul of the agarose and cell suspension to the inside surface of the cover slip. Carefully use the pipette tip to easily lay the slip down onto the slide (no bubbles).

(3) Place slide on ice (minimum of three minutes).

(4) Gently slide off the coverslip and add a third agarose layer consisting of 75 ul LMPA to the slide. Replace coverslip and return to ice tray.

VI. LYSIS

- Remove coverslip and slowly lower slide into cold, freshly made Lysing Solution.

Protect from light and place in 4 degrees Celsius refrigeration for a minimum of 3 hours.

Slides may be stored for extended periods of time in cold Lysing Solution (at least 4 weeks) without affecting migration.

VII. ELECTROPHORESIS AND NEUTRALIZATION

(1) Make sure the electrophoresis tank is clean, rinse in distilled water.

(2) Gently remove the slides from the Lysing Solution. Place slides on the horizontal gel box near the anode (+) end, sliding them as close together as possible. Always work with 24 slides at the time, if less you have to fill in the empty space in the electrophoresis tank with blank slides.

(5) Fill the buffer reservoirs with freshly made Electrophoresis Buffer until liquid level completely covers the slides (avoid bubbles over the slides).

(6) Let slides sit in the alkaline buffer for 20 minutes to allow unwinding of the DNA and the expression of different classes of alkali-labile damage.

(7) Turn on power supply to 25 volts and adjust current to 300 mAmp. by slowly raising or lowering the buffer level. Depending on the purpose of the study and the extent of migration in control samples, allow slides to run for 10 to 40 minutes.

- (8) Turn off the power. Gently lift the slides from the buffer and place on a staining tray. Dropwise coat the slides with Neutralization Buffer, let sit for at least 5 minutes. Repeat two more times.
 - (9) Drain slides, add 50 ul 1x Ethidium Bromide stain and cover with a fresh coverslip.
 - (10) Before viewing the slide, blot away excess liquid on the back and edges.
- Note: During the electrophoresis process work under dimmed lights.

IX. EVALUATION OF DNA DAMAGE

- (1) For visualization of DNA damage, observations are made of ethidium bromide stained DNA at 40x magnification using a fluorescent microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm.
- (2) We use Confocal Comet or Leca Quantiment 520 image analysis system linked to CCD camera to quantitate DNA image length, DNA image diameter, nuclear diameter, tail intensity, and tail moment. Generally, 50 randomly selected cells per sample are analyzed.